agonistic effects. The reaction products were separated by SDS-PAGE, followed by Coomassie Blue or silver staining. *Treatment of cartilage explants with HtrA1:* Human osteoarthritic cartilage explants were treated with recombinant HtrA1, with or without CPII. Conditioned media were assayed for sGAG release by DMMB. Proteoglycans in the media were precipitated using 1% cetylpyridinium chloride and subjected to SDS-PAGE followed by Coomassie Blue or silver staining.

Results: The cartilage proteoglycan aggrecan was identified as one of the potential substrates of HtrA1 in the mass spectrometry-based "degradomics" analysis. Incubation of recombinant aggrecan G1-IGD-G2 and IGD constructs with wild-type HtrA1, but not mutant HtrA1, resulted in distinct cleavage of these substrates. HtrA1 activity was further enhanced by the peptide agonist CPII, and inhibited by the HtrA inhibitor Ucf-101. In addition, recombinant HtrA1 cleaved native human aggrecan in the presence of the CPII peptide agonist. Treatment of cartilage explants with recombinant HtrA1 significantly increased ($p<0.05$) the amount of sGAG released compared to control. Further, the addition of CPII significantly increased ($p<0.05$) the amount of sGAG release compared to treatment with HtrA1 alone.

Conclusions: Our data suggest that the collagen type II C-propeptide may induce proteoglycan catabolism by stimulating the protease activity of HtrA1. Elevated levels of collagen type II C-propeptide have been detected in osteoarthritic human articular cartilage, due to increased collagen synthesis. Excessive HtrA1 protease activity in OA cartilage may represent another contributing factor in OA disease progression.

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CRUCIAL ROLE OF VISFATIN/PBEF IN MATRIX DEGRADATION AND PGE_2 SYNTHESIS IN CHONDROCYTES: POSSIBLE INFLUENCE ON OSTEOARTHRITIS

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Purpose: To evaluate the contribution of visfatin, an adipose tissue-derived hormone, to the pathophysiology of osteoarthritis (OA) by examining its role in prostaglandin E_2 (PGE_2) synthesis and matrix degradation. In an inflammatory context, PGE_2 synthesis is catalysed by cyclooxygenase type 2 (COX-2) and microsomal prostaglandin E synthase type 1 (mPGES-1), whereas NAD+-dependent 15-hydroxy prostaglandin dehydrogenase (15-PGDH) degrades PGE_2.

Methods: The synthesis of visfatin by human chondrocytes from OA patients, with and without stimulation with interleukin-1β (IL-1β- 10 ng/ml), was assessed by real-time RT-PCR and immunoblotting. The effects of visfatin (1 to 10μg/ml) on mPGES-1, 15-PGDH, PGE_2, MMP-3 and MMP-13 expressions by human OA chondrocytes and by primary mouse articular chondrocytes were examined by quantitative RT-PCR, immunoblotting and ELISA. A siRNA strategy was used to assess the influence of visfatin on the IL-1β induced release of PGE_2. Finally, the role of IGF-1R in visfatin signalling was studied using primary chondrocytes from IGF-1R knockout mice ($IGF\text{-}1R^{-/-}$).

Results: (1) Visfatin was constitutively expressed by cultured human OA chondrocytes. Its expression increased 6-fold in response to 10 ng/ml IL-1β ($p<0.05$).

(2) Visfatin at 1 to 5 μg/ml triggered MMP-3 and MMP-13 mRNA expression (up to 6-fold, $p<0.01$) by primary mouse articular chondrocytes. Stimulation with 5 μg/ml visfatin led to a release of 572 ± 280 ng/ml MMP-3 protein ($p<0.05$). Visfatin also induced i) PGE_2 release (controls 47 ± 8 versus 141 ± 10 pg/ml when treated with 10 μg/ml visfatin, $p<0.05$), ii increased expression of the mPGES-1 (14-fold increase, $p<0.01$) and iii) a 90% decrease ($p<0.05$) of the 15-PGDH. Interestingly, 1 ng/ml IL-1β plus visfatin (1, 2.5 or 5 μg/ml for 24 hours) had additive effects on PGE_2 release (19-fold, 31-fold and 35-fold compared to IL-β, $p<0.05$; [1 ng/ml IL-β released 1506 ± 67 pg/ml]). Moreover, IL-1β dramatically decreased 15-PGDH expression by 95% ($p<0.001$).

(3) Blocking visfatin expression by siRNA inhibited IL-1β-induced PGE_2 release: triggered the release of (1430 ± 467 pg/ml in presence of IL-1β (10ng/ml) versus 985 ± 292 pg/ml in presence of IL-1β + siRNA visfatin, -35%, $p<0.01$) probably due to a 40% inhibition of mPGES-1 expression ($p<0.01$). (4) Visfatin is known to bind to, and to activate insulin receptor (IR). However, IR is not considered to be usually present on chondrocytes. We therefore tested the implication of IGF-1R, a close homologue to IR, in visfatin signalling. When stimulated with 5 μg/ml visfatin, $IGF\text{-}1R^{-/-}$ chondrocytes unexpectedly exhibited higher PGE_2 release than $IGF1R^{+/+}$ controls (228 ± 4 compared to 86 ± 29 pg/ml, $p<0.05$) which rules out the direct implication of IGF-1R in visfatin action.

Conclusions: Visfatin triggers the synthesis and the release of MMP-3 and MMP-13 and induces PGE_2 synthesis resulting from an increase of mPGES-1 and a decrease of 15-PGDH expression in chondrocytes. We therefore consider that visfatin is a novel and a potential critical target for OA. In vivo experiments are now needed to test this hypothesis.

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AN EXPERIMENTAL MODEL TO STUDY THE MECHANISMS OF EPIGENETIC DNA DE-METHYLATION OBSERVED IN HUMAN OSTEOARTHRITIS

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Purpose: Previous studies (Arthritis Rheum 52:3110-24) showed that DNA de-methylation at specific CpG sites in the promoters was associated with the abnormal synthesis of matrix-degrading enzymes in human osteoarthritis. However, it is not known whether DNA de-methylation actually causes the abnormal expression of the proteases. To demonstrate possible cause-effect relationships and to study the mechanisms involved in the loss of DNA methylation requires an *in vitro* system in which experimentally induced gene induction is correlated with de-methylation at specific CpG sites.

Methods: Since monolayer cultures of articular chondrocytes are an established model to study the induction of the typical OA proteases by inflammatory cytokines, we used this system. Healthy chondrocytes were harvested from human femoral head cartilage after hemi-arthroplasty following a fracture of the neck of femur. The chondrocytes from each patient were divided into five groups: non-culture; control culture; culture with the de-methylating agent 5-aza-deoxycytidine (5-aza-dC) or the inflammatory cytokines IL-1β or TNF-α/oncostatin M. At confluency (4-5 weeks), total RNA and genomic DNA were extracted simultaneously. Relative mRNA expression was quantified by SybrGreen-based real-time PCR and a method for quantifying the percent of cells with DNA methylation at one specific CpG site was developed (Epigenetics 2: 86-95). ELISA was used to analyze IL-1β in the culture.

Results: Initial non-quantitative experiments confirmed IL-1β - induced expression of MMP-3 and MMP-13 and also demonstrated induction of IL-1β by itself, which correlated with loss of

DNA methylation in the IL-1β promoter in 3/5 patients. Further work concentrated on this gene. Bisulfite modification suggested that the CpG sites at -290bp and - 247bp were important for the epigenetic regulation of IL-1β, and the site at -290bp was selected for quantification of DNA methylation. We then determined which experimental conditions resulted in the greatest loss of DNA methylation and whether this paralleled the increase in mRNA expression. Culture with the de-methylating factor 5-aza-dC halved DNA methylation, which resulted in 4-8 fold increases in IL-1β expression compared with cultured controls. This demonstrated that DNA de-methylation *per se* increased gene expression. However, this was not the only factor involved, since far greater effects were seen with the inflammatory cytokines: IL-1β reduced methylation to ~15%, cresulting in 50-100 fold increases in gene expression. The greatest effect was seen with TNF-α/OSM, which abolished DNA methylation almost completely and caused IL-1β expression to increase 500-1000 fold. The cytokine-induced expression was translated into protein. Unlike IL-1β, TNF-α expression was not susceptible to auto-induction or epigenetic control.

Conclusions: A system has been developed in which loss of DNA methylation in combination with 100-1000 fold increases in gene expression can be induced experimentally - a crucial prerequisite for mechanistic studies. Moreover, we demonstrated for the first time that inflammatory cytokines can cause loss of DNA methylation in addition to all other known effects, and that IL-1β (but not TNF-α) induces its own expression.

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MECHANISMS OF ACTION OF IKKα IN REGULATING THE HYPERTROPHIC TRANSITION OF PRIMARY OSTEOARTHRITIC CHONDROCYTES

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Purpose: Our previous work revealed differential roles for the NF-κB activating kinases in controlling the progression towards terminal differentiation of primary osteoarthritic chondrocytes. Here we elaborate some of the multiple steps whereby IKKα controls the terminal chondrogenic differentiation program.

Methods: Primary chondrocytes were derived from 15 Osteoarthritis (OA) patients undergoing joint arthroplasty. IKKα or IKKβ shRNAs were stably expressed by pSuper retroviral transduction of IKKα or IKKβ specific shOligos followed by selection of puromycin resistant cells and KD efficiencies were verified by immunoblotting. High density monolayer and micromass cultures under mineralizing conditions were selected to investigate the mechanisms whereby the IKKs control chondrocyte terminal differentiation. Runx2 was evaluated by both REAL TIME PCR in 1 week old micromasses and by immunohistochemistry in 3 week old micromasses and Sox9 expression in high density monolayer cultures by immunoblotting. The effects of IKKα KD on type 2 collagen accumulation at the transcriptional or post-translational levels were investigated in micromasses. ECM remodelling was directly evaluated by staining for neoepitopes (Col2 3/4 C and DIPEN and NITEGE). Moreover, IKKα expression was rescued in IKKα KD chondrocytes with either a wild type or kinase-dead IKKα(K44M) mutant to explore the connection between IKKα's effects on chondrogenesis and the NF-κB pathway.

Results: IKK KDs were at least 80% and generally greater than 90%. Both IKK KD were found associated with increased Col2 mRNA expression and also resulted in defective ECM remodelling as shown by IHC detection of neoepitopes. Silencing IKKβ markedly increased sGAG accumulation, in conjunction with increased Sox9 expression. However, IKKα ablation markedly enhanced collagen 2 deposition independently of Sox9 but instead in association with a strong remodelling block in conjunction with Runx2 suppression. In addition ablation of either IKKα or IKKβ also inhibited Collagen X deposition thus providing additional evidence, along with blocks in calcium deposition, that each IKK (albeit via different mechanisms) is of central importance for progression towards terminal differentiation.

Conclusions: We have uncovered an unexpected, pivotal role for IKKα in the terminal chondrogenesis of primary osteoarthritic human chondrocytes. IKKα controls the transition to the hypertrophic phase at multiple levels, while IKKβ appears to mimick the effects of canonical NF-κB activation. Preliminary indications of an IKKα rescue experiment suggest that its more profound effects could be independent of the NF-κB pathway. In conjunction with the healthier status of IKKα KD chondrocytes, with respect to their extensive ECM and high viability, our results support the view that IKKα could be a novel therapeutic target for Osteoarthritis with its ablation causing a profound blockage towards terminal differentiation.

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APOLIPOPROTEIN A-1 IS A NOVEL INDUCER OF AGGRECAN BREAKDOWN IN CULTURED ARTICULAR CARTILAGE

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Purpose: While investigating the effects of sharp injury upon inflammatory signalling pathways in articular cartilage, we found that synovial fluid strongly activated MAP kinases in cartilage explants adapted to culture. We set out to identify the activator and investigate its effects on chondrocyte function.

Methods: Cartilage from porcine metacarpophalangeal joints was cultured in serum-free medium for 24h before treatment with porcine synovial fluid. Tissue extracts were analysed for MAPK (ERK, p38 and JNK) and ATF-2 (a preferred JNK substrate) activation by phospho-Western blotting. Culture medium samples were analysed for the presence of aggrecanase-generated aggrecan fragments by using antibodies against the aggrecan ARGS neoepitope. Porcine synovial fluid was subjected to sequential chromatography (Resource Q, Resource S and Blue Sepharose) and the MAPK activator was identified by mass-spectrometry.

Results: The porcine synovial fluid activator was identified as *apolipoprotein A-1* (*APOA-1*) the major component of high density lipoprotein (*HDL*) in tissue fluids. Porcine APOA-1 purified from synovial fluid and commercially available human APOA-1 activated MAPKs and also induced aggrecan breakdown in cultured cartilage. APOA-1 induced mRNA of *ADAMTS-4* and *-5* and *MMP-13*. APOA-1 differed from typical inflammatory mediators that cause cartilage catabolism (IL-1, TNF, LPS), in that it did not induce cyclooxygenase-2 (COX-2), a typical inflammatory response gene.

Conclusions: APOA-1 in synovial fluid causes MAPK activation and induces aggrecanases in cartilage. It appears to cause a matrix-resorbing response without inducing inflammatory response genes. APOA-1 (apparent Mr ~120kD) may normally be excluded from the cartilage matrix but is perhaps able to access